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(54) **Title:** HUMAN GENOME-DERIVED SINGLE EXON NUCLEIC ACID PROBES USEFUL FOR ANALYSIS OF GENE EXPRESSION IN HUMAN HEART

(57) **Abstract:** A single exon nucleic acid microarray comprising a plurality of single exon nucleic acid probes for measuring gene expression in a sample derived from human heart is described. Also described are single exon nucleic acid probes expressed in the heart and their use in methods for detecting gene expression.

HUMAN GENOME-DERIVED SINGLE EXON NUCLEIC ACID PROBES USEFUL FOR ANALYSIS OF GENE EXPRESSION IN HUMAN HEART

CROSS REFERENCE TO RELATED APPLICATIONS

The present application is a continuation-in-part of U.S. patent application serial nos. 09/632,366, filed August 3, 2000 and 09/608,408, filed June 30, 2000; claims the benefit under 35 U.S.C. s 119(e) of U.S.provisional patent application serial nos. 60/236,359, filed September 27, 2000, 60/234,687, filed September 21, 2000, 60/207,456, filed May 26, 2000, and 60/180,312, filed February 4, 2000; and further claims the benefit under 35 U.S.C. s 119(a) of UK patent application no. 0024263.6, filed October 4, 2000, the disclosures of which are incorporated herein by reference in their entireties.

REFERENCE TO SEQUENCE LISTING AND INCORPORATION BY REFERENCE THEREOF

The present application includes a Sequence Listing in electronic format, filed pursuant to PCT Administrative Instructions 801 - 806 on a single CD-R disc, in triplicate, containing a file named pto_HEART.txt, created 24 January 2001, having 20,186,946 bytes. The Sequence Listing contained in said file on said disc is incorporated herein by reference in its entirety.

Field of the Invention

The present invention relates to genome-derived single exon microarrays useful for verifying the expression of regions of genomic DNA predicted to encode protein. In particular, the present invention relates to unique genome-derived single exon nucleic acid probes expressed in human heart and single exon nucleic acid microarrays that include such probes.

Background of the Invention

For almost two decades following the invention of general techniques for nucleic acid sequencing, Sanger *et al., Proc. Natl. Acad. Sci. USA* 70(4):1209-13 (1973); Gilbert *et al., Proc. Natl. Acad. Sci. USA* 70(12):3581-4 (1973), these techniques were used principally as tools to further the understanding of proteins – known or suspected – about which a basic foundation of biological knowledge had already been built. In many cases, the cloning effort that preceded sequence identification had been both informed and directed by that antecedent biological understanding.

For example, the cloning of the T cell receptor for antigen was predicated upon its known or suspected cell type-specific expression, by its suspected membrane association, and by the predicted assembly of its gene via T cell-specific somatic recombination. Subsequent sequencing efforts at once confirmed and extended understanding of this family of proteins. Hedrick *et al., Nature* 308(5955):153-8 (1984).

More recently, however, the development of high throughput sequencing methods and devices, in concert with large public and private undertakings to sequence the human and other genomes, has altered this investigational paradigm: today, sequence information often precedes understanding of the basic biology of the encoded protein product.

One of the approaches to large-scale sequencing is predicated upon the proposition that expressed sequences – that is, those accessible through isolation of mRNA – are of greatest initial interest. This "expressed sequence tag" ("EST") approach has already yielded vast amounts of sequence data (see for example Adams *et al.*, *Science* 252:1651 (1991); Williamson, *Drug Discov. Today* 4:115 (1999)). For nucleic acids sequenced by this approach, often the only biological information that is known *a priori* with any certainty is the likelihood of biologic expression itself. By virtue of the species and tissue from which the mRNA had originally been obtained, most such sequences are also annotated with the identity of the species and at least one tissue in which expression appears likely.

More recently, the pace of genomic sequencing has accelerated dramatically. When genomic DNA serves as the initial substrate for sequencing efforts, expression cannot be presumed; often the only *a priori* biological information about the sequence includes the species and chromosome (and perhaps chromosomal map location) of origin.

With the ever-accelerating pace of sequence accumulation by directed, EST, and genomic sequencing approaches — and in particular, with the accumulation of sequence information from multiple genera, from multiple species within genera, and from multiple individuals within a species — there is an increasing need for methods that rapidly and effectively permit the functions of nucleic sequences to be elucidated. And as such functional information accumulates, there is a further need for methods of storing such functional information in meaningful and useful relationship to the sequence itself; that is, there is an increasing need for means and apparatus for annotating raw sequence data with known or predicted functional information.

Although the increase in the pace of genomic sequencing is due in large part to technological changes in sequencing strategies and instrumentation, Service, *Science* 280:995 (1998); Pennisi, *Science* 283: 1822-1823 (1999), there is an important functional motivation as well.

While it was understood that the EST approach would rarely be able to yield sequence information about the noncoding portions of the genome, it now also appears the EST approach is capable of capturing only a fraction of a genome's actual expression complexity.

For example, when the *C. elegans* genome was fully sequenced, gene prediction algorithms identified over 19,000 potential genes, of which only 7,000 had been found by EST sequencing. *C. elegans* Sequencing Consortium, *Science* 282:2012 (1998). Analogously, the recently completed sequence of chromosome 2 of *Arabidopsis* predicts over 4000 genes, Lin *et al.*, *Nature*, 402:761 (1999), of which only about 6% had previously been identified via EST sequencing efforts. Although the human genome has the greatest depth of EST coverage, it is still woefully short of surrendering all of its genes. One recent estimate suggests that the human genome contains more than 146,000 genes, which would at this point leave greater than half of the genes undiscovered. It is now predicted that many genes, perhaps 20 to 50%, will only be found by genomic sequencing.

There is, therefore, a need for methods that permit the functional regions of genomic sequence — and most importantly, but not exclusively, regions that function to encode genes — to be identified.

Much of the coding sequence of the human genome is not homologous to known genes, making detection of open reading frames ("ORFs") and predictions of gene function difficult. Computational methods exist for predicting coding regions in eukaryotic genomes. Gene prediction programs such as GRAIL and GRAIL II, Uberbacher *et al.*, *Proc. Natl. Acad. Sci. USA* 88(24):11261-5 (1991); Xu *et al.*, *Genet. Eng.* 16:241-53 (1994); Uberbacher *et al.*, *Methods Enzymol.* 266:259-81 (1996); GENEFINDER, Solovyev *et al.*, *Nucl. Acids. Res.* 22:5156-63 (1994); Solovyev *et al.*, *Ismb* 5:294-302 (1997); and GENESCAN, Burge *et al., J. Mol. Biol.* 268:78-94 (1997), predict many putative genes without known homology or function. Such programs are known, however, to give high false positive rates. Burset *et al., Genomics* 34:353-367 (1996). Using a consensus obtained by a plurality of such programs is known to increase the reliability of calling exons from genomic sequence. Ansari-Lari *et al., Genome Res.* 8(1):29-40 (1998)

Identification of functional genes from genomic data remains, however, an imperfect art. For example, in reporting the full sequence of human chromosome 21, the Chromosome 21 Mapping and Sequencing Consortium reports that prior bioinformatic estimates of human gene number may need to be revised substantially downwards. *Nature* 405:311-199 (2000); Reeves, *Nature* 405:283-284 (2000).

Thus, there is a need for methods and apparatus that permit the functions of the regions identified bioinformatically — and specifically, that permit the expression of regions predicted to encode protein — readily to be confirmed experimentally.

Recently, the development of nucleic acid microarrays has made possible the automated and highly parallel measurement of gene expression. *Reviewed in* Schena (ed.), DNA Microarrays : A Practical Approach (Practical Approach Series), Oxford University Press (1999) (ISBN: 0199637768); *Nature Genet.* 21(1)(suppl):1 - 60 (1999); Schena (ed.), Microarray Biochip: Tools and Technology, Eaton Publishing Company/BioTechniques Books Division (2000) (ISBN: 1881299376).

It is common for microarrays to be derived from cDNA/EST libraries, either from those previously described in the literature, such as those from the I.M.A.G.E. consortium, Lennon *et al., Genomics* 33(1):151-2 (1996), or from the construction of "problem specific" libraries targeted at a particular biological question, R.S. Thomas *et al.*, *Cancer Res.* (in press). Such microarrays by definition can measure expression only of those genes found in EST libraries, and thus have not been useful as probes for genes discovered solely by genomic sequencing.

The utility of using whole genome nucleic acid microarrays to answer certain biological questions has been demonstrated for the yeast *Saccharomyces cerevisiae*. De Risi *et al.*, *Science* 278:680 (1997). The vast majority of yeast nuclear genes, approximately 95% however, are single exon genes, *i.e.*, lack introns, Lopez *et al.*, *RNA* 5:1135-1137 (1999); Goffeau *et al.*, *Science* 274:563-67 (1996), permitting coding regions more readily to be identified. Whole genome nucleic acid microarrays have not generally been used to probe gene expression from more complex eukaryotic genomes, and in particular from those averaging more than one intron per gene.

Diseases of the heart and vascular system are a significant cause of human morbidity and mortality. Increasingly, genetic factors are being found that contribute to predisposition, onset, and/or aggressiveness of most, if not all, of these diseases. Although mutations in single genes have on occasion been identified as causative, these disorders are for the most part believed to have polygenic etiologies. There is a need for methods and apparatus that permit prediction, diagnosis and prognosis of diseases of the human heart, particularly those diseases with polygenic etiology.

Summary of the Invention

The present invention solves these and other problems in the art by providing methods and apparatus for predicting, confirming, and displaying functional information derived from genomic sequence. The present invention also provides apparatus for verifying the expression of putative genes identified within genomic sequence.

In particular, the invention provides novel genome-derived single exon nucleic acid microarrays useful for verifying the expression of putative genes identified within genomic sequence.

The present invention also provides compositions and kits for the ready production of nucleic acids identical in sequence to, or substantially identical in sequence to, probes on the genome-derived single exon microarrays of the present invention.

Accordingly, in a first aspect of the invention, there is provided a spatially-addressable set of single exon nucleic acid probes for measuring gene expression in a sample derived from human heart, comprising a plurality of single exon nucleic acid probes according to any one of the nucleotide sequences set out in SEQ ID NOs: 1 - 9,980 or a complementary sequence, or a portion of such a sequence.

By plurality is meant at least two, suitably at least 20, most suitably at least 100, preferably at least 1000 and, most preferably, upto 5000.

In one embodiment of the first aspect, each of said plurality of probes is separately and addressably amplifiable.

In an alternative embodiment, each of said plurality of probes is separately and addressably isolatable from said plurality.

In a preferred embodiment, each of said plurality of probes is amplifiable using at least one common primer. Preferably, each of said plurality of probes is amplifiable using a first and a second common primer.

In yet another embodiment, said set of single exon nucleic acid probes comprises between 50 - 20,000 probes, for example, 50 - 5000.

Suitably, said set of single exon nucleic acid probes comprises at least 50 - 1000 discrete single exon nucleic acid probes having a sequence as set out in any of SEQ ID NOS.: 1 - 19,771 or a complimentary sequence, or a portion of such a sequence.

Preferably, the average length of the single exon nucleic acid probes is between 200 and 500 bp. It is preferred that the average length should be at least 200bp, suitably at least 250bp, most suitably at least 300bp, preferably at least 400bp and, most preferably, 500 bp.

In another embodiment, the single exon nucleic acid probes lack prokaryotic and bacteriophage vector sequence. It is preferred that at least 50%, suitably at least 60%, most suitably at least 70%, preferably at least 75%, more preferably at least 80, 85, 90, 95 or 99% of said single exon nucleic acid probes lack prokaryotic and bacteriophage vector sequence.

In another preferred embodiment, said single exon nucleic acid lack homopolymeric stretches of A or T. It is preferred that at least 50%, suitably at least 60%, most suitably at least 70%, preferably at least 75%, more preferably at least 80, 85, 90, 95 or 99% of said single exon nucleic acid probes lack homopolymeric stretches of A or T.

Preferably, a spatially-addressable set of single exon nucleic acid probes in accordance with the first aspect of the invention is is addressably disposed upon a substrate.

Suitable substrates include a filter membrane which may, preferably, be nitrocellulose or nylon. The nylon may preferably, be positively-charged. Other suitable substrates include glass, amorphous silicon, crystalline silicon, and plastic. Further suitable materials include polymethylacrylic, polyethylene, polypropylene, polyacrylate, polymethylmethacrylate, polyvinylchloride, polytetrafluoroethylene, polystyrene, polycarbonate, polyacetal, polysulfone, celluloseacetate, cellulosenitrate, nitrocellulose, and mixtures thereof.

In a second aspect of the invention, there is provided a microarray comprising a spatially addressable set of single exon nucleic acid probes in accordance with the first aspect of the invention.

In one embodiment, a genome-derived single-exon microarray is packaged together with such an ordered set of amplifiable probes corresponding to the probes, or one or more subsets of probes, thereon. In alternative embodiments, the ordered set of amplifiable probes is packaged separately from the genome-derived single exon microarray.

In another aspect, the invention provides genome-derived single exon nucleic acid probes useful for gene expression analysis, and particularly for gene expression analysis by microarray. In particular embodiments of this aspect, the present invention provides human single-exon probes that include specifically-hybridizable fragments of SEQ ID Nos. 9,981 - 19,771, wherein the fragment hybridizes at high stringency to an expressed human gene. In particular embodiments, the invention provides single exon probes comprising SEQ ID Nos. 1 - 9,980.

Accordingly, in a third aspect of the invention, there is provided a single exon nucleic acid probe for measuring human gene expression in a sample derived from human heart which is a nucleic acid molecule comprising a nucleotide sequence as set out in any of SEQ ID NOs.: 1 - 9,980 or a complementary sequence or a fragment thereof wherein said probe hybridizes at high stringency to a nucleic acid expressed in the human heart.

In one embodiment, a single exon nucleic acid probe in accordance with the third aspect comprises a nucleotide sequence as set out in any of SEQ ID NOs.: 9,981 - 19,771 or a complementary sequence or a fragment thereof.

In a fourth aspect of the invention, there is provided a single exon nucleic acid probe for measuring human gene expression in a sample derived from human heart which is a nucleic acid molecule having a sequence encoding a peptide comprising a peptide sequence as set out in any of SEQ ID NOs.: 19,772 - 29,119 or a complementary sequence or a fragment thereof wherein said probe hybridizes at high stringency to a nucleic acid expressed in the human heart.

Preferably, a single exon nucleic acid probe in accordance with the third or fourth aspects of the invention comprises between at least 15 and 50 contiguous nucleotides of said SEQ ID NO:. It is preferred that the single exon nucleic acid probe comprises at least 15, suitably at least 20, more suitably at least 25 or preferably at least 50 contiguous nucleotides of said SEQ ID NO:.

In another preferred embodiment, a single exon nucleic acid probe in accordance with the third or fourth aspects of the invention is between 3kb and 25kb in length. It is preferred that said probe is no more than 3kb, suitably no more than 5kb, more suitably no more than 10kb, preferably 15kb, more preferably 20kb or, most preferably, no more than 20kb in length.

Preferably, a single exon nucleic acid probe in accordance with either the fifth or sixth aspect of the invention is DNA, preferably single-stranded DNA, RNA or PNA.

In another embodiment of either the third or fourth aspect of the invention, a single exon nucleic acid probe is detectably labeled. Suitable detectable labels include a radionuclide, a fluorescent label or a first member of a specific binding pair. Suitable fluorescent labels include dyes such as cyanine dyes, preferably Cy3 and Cy5 although other suitable dyes will be known to those skilled in the art.

In a particularly preferred embodiment, a single exon nucleic acid probe in accordance with either the third or fourth aspect of the invention lacks prokaryotic and bacteriophage vector sequence. In yet another embodiment, a single exon nucleic acid probe in accordance with either the third or fourth aspect of the invention lacks homopolymeric stretches of A or T.

In a fifth aspect of the invention, there is provided an amplifiable nucleic acid composition, comprising:

the single exon nucleic acid probe in accordance with either of the third or fourth aspects of the invention; and at least one nucleic acid primer;

wherein said at least one primer is sufficient to prime enzymatic amplification of said probe.

In an sixth aspect of the invention, there is provided a method of measuring gene expression in a sample derived from human heart, comprising:

contacting the single exon microarray in accordance with the second aspect of the invention, with a first collection of detectably labeled nucleic acids, said first collection of nucleic acids derived from mRNA of human heart; and then

measuring the label detectably bound to each probe of said microarray.

In a seventh aspect of the invention, there is provided a method of identifying exons in a eukaryotic genome, comprising:

algorithmically predicting at least one exon from genomic sequence of said eukaryote; and then

detecting specific hybridization of detectably labeled nucleic acids to a single exon probe,

wherein said detectably labeled nucleic acids are derived from mRNA from the heart of said eukaryote, said probe is a single exon probe having a fragment identical in sequence to, or complementary in sequence to, said predicted exon, said probe is included within a single exon microarray in accordance with the first aspect of the invention, and said fragment is selectively hybridizable at high stringency.

In a eighth aspect of the invention, there is provided a method of assigning exons to a single gene, comprising:

identifying a plurality of exons from genomic sequence in accordance with the seventh aspect of the invention; and then

measuring the expression of each of said exons in a plurality of tissues and/or cell types using hybridization to single exon microarrays having a probe with said exon,

wherein a common pattern of expression of said exons in said plurality of tissues and/or cell types indicates that the exons should be assigned to a single gene.

In an ninth aspect of the invention, there is provided a nucleic acid sequence as set out in any of SEQ ID NOs: 1 - 19,771 wherein said sequence encodes a peptide.

In a tenth aspect of the invention, there is provided a peptide encoded by a sequence comprising a sequence as set out in any of SEQ ID NOs: 9,981 - 19,771, or a complementary sequence or coding portion thereof.

In a preferred embodiment, a peptide may be encoded by a sequence comprising a sequence set out in any of SEQ ID NOS.: 1 - 9,980.

In a further aspect, the invention provides peptides comprising an amino acid sequence translated from the DNA fragments, said amino acid sequences comprising SEQ ID NOS.: 9,981 - 19,771.

Accordingly in a eleventh aspect of the invention there is provided a peptide comprising a sequence as set out in any of SEQ ID NOs: 19,772 - 29,119, or fragment thereof.

In another aspect, the invention provides means for displaying annotated sequence, and in particular, for displaying sequence annotated according to the methods and apparatus of the present invention. Further, such display can be used as a preferred graphical user interface for electronic search, query, and analysis of such annotated sequence.

Detailed Description of the Invention

Definitions

As used herein, the term "microarray" and phrase "nucleic acid microarray" refer to a substrate-bound collection of plural nucleic acids, hybridization to each of the plurality of bound nucleic acids being separately detectable. The substrate can be solid or porous, planar or non-planar, unitary or distributed.

As so defined, the term "microarray" and phrase "nucleic acid microarray" include all the devices so called in Schena (ed.), DNA Microarrays: A Practical Approach (Practical Approach Series), Oxford University Press (1999) (ISBN: 0199637768); *Nature Genet.* 21(1)(suppl):1 - 60 (1999); and Schena (ed.), Microarray Biochip: Tools and Technology, Eaton Publishing Company/BioTechniques Books Division (2000) (ISBN: 1881299376). As so defined, the term "microarray" and phrase "nucleic acid microarray" further include substrate-bound collections of plural nucleic acids in which the nucleic acids are distributably disposed on a plurality of beads, rather than on a unitary planar substrate, as is described, *inter alia*, in Brenner *et al.*, *Proc. Natl. Acad. Sci. USA* 97(4):166501670 (2000); in such case, the term "microarray" and phrase "nucleic acid microarray" refer to the plurality of beads in aggregate.

As used herein with respect to a nucleic acid microarray, the term "probe" refers to the nucleic acid that is, or is intended to be, bound to the substrate; in such context, the term "target" thus refers to nucleic acid intended to be bound thereto by Watson-Crick complementarity. As used herein with respect to solution phase hybridization, the term "probe" refers to the nucleic acid of known sequence that is detectably labeled.

As used herein, the expression "probe comprising SEQ ID NO.", and variants thereof, intends a nucleic acid probe, at least a portion of which probe has either (i) the sequence directly as given in the referenced SEQ ID NO., or (ii) a sequence complementary to the sequence as given in the referenced SEQ ID NO., the choice as between sequence directly as given and complement thereof dictated by the requirement that the probe hybridize to mRNA.

As used herein, the term "open reading frame" and the equivalent acronym "ORF" refer to that portion of an exon that can be translated in its entirety into a sequence of contiguous amino acids i.e. a nucleic acid sequence that, in at least one reading frame, does not possess stop codons; the term does not require that the ORF encode the entirety of a natural protein.

As used herein, the term "amplicon" refers to a PCR product amplified from human genomic DNA, containing the predicted exon.

As used herein the term "exon" refers to the consensus prediction of the various exon and gene predicting algorithms i.e. a nucleic acid sequence bioinformatically predicted to encode a portion of a natural protein.

As used herein, the term "peptide" refers to a sequence of amino acids. The sequences referred to as

PEPTIDE SEQ ID NOS.: are the predicted peptide sequences that would be translated from one of the exons, or a portion thereof set out in exon SEQ ID NOS.:. The codons encoding the peptide are wholly contained within the exon.

As used herein, a "portions" of a defined nucleotide sequence or sequences can be and, preferably, are fragments unique to that sequence or to one or a combination of those sequences. A fragment unique to a nucleic acid molecule is one that is a signature for the larger nucleic acid molecule.

As used herein, the phrase "expression of a probe" and its linguistic variants means that the ORF present within the probe, or its complement, is present within a target mRNA.

As used herein, "stringent conditions" refers to parameters well known to those skilled in the art. When a nucleic acid molecule is said to be hybridisable to another of a given sequence under "stringent conditions" it is meant that it is homologous to the given sequence.

As used herein, the phrase "specific binding pair" intends a pair of molecules that bind to one another with high specificity. Binding pairs are said to exhibit specific binding when they exhibit avidity of at least 10^7, preferably at least 10^8, more preferably at least 10^9 liters/mole. Nonlimiting examples of specific binding pairs are: antibody and antigen; biotin and avidin; and biotin and streptavidin.

As used herein with respect to the visual display of annotated genomic sequence, the term "rectangle" means any geometric shape that has at least a first and a second border, wherein the first and second borders each are capable of mapping uniquely to a point of another visual object of the display.

As used herein, a "Mondrian" means a visual display in which a single genomic sequence is annotated with predicted and experimentally confirmed functional information.

Brief Description of the Drawings

The present invention is further illustrated with reference to the following non-limiting figures and examples in which:

FIG. 1 illustrates a process for predicting functional regions from genomic sequence, confirming the functional activity of such regions experimentally, and associating and displaying the data so obtained in meaningful and useful relationship to the original sequence data;

FIG. 2 further elaborates that portion of the process schematized in FIG. 1 for predicting functional regions from genomic sequence;

FIG. 3 illustrates a Mondrian visual display;

FIG. 4 presents a Mondrian showing a hypothetical annotated genomic sequence;

FIG. 5 is a histogram showing the distribution of ORF length and PCR products as obtained, with ORF length shown in black and PCR product length shown in dotted lines;

FIG. 6 is a histogram showing the distribution, among exons predicted according to the methods described, of expression as measured using simultaneous two color hybridization to a genome-derived single exon microarray. The graph shows the number of sequence-verified products that were either not expressed ("0"), expressed in one or more but not all tested tissues ("1" - "9"), or expressed in all tissues tested ("10");

FIG. 7 is a pictorial representation of the expression of verified sequences that showed expression with signal intensity greater than 3 in at least one tissue, with: FIG. 7A showing the expression as measured by microarray hybridization in each of the 10 measured tissues, and the expression as measured "bioinformatically" by query of EST, NR and SwissProt databases; with FIG. 7B showing the legend for display of physical expression (ratio) in FIG. 7A; and with FIG. 7C showing the legend for scoring EST hits as depicted in FIG. 7A;

FIG. 8 shows a comparison of normalized CY3 signal intensity for arrayed sequences that were identical to sequences in existing EST, NR and SwissProt databases or that were dissimilar (unknown), where black denotes the signal intensity for all sequence-verified products with a BLAST Expect ("E") value of greater than 1e-30 (1 x 10^{-30}) ("unknown") and a dotted line denotes sequence-verified spots with a BLAST expect ("E") value of less than 1e-30 (1 x 10^{-30})("known");

FIG. 9 presents a Mondrian of BAC AC008172 (bases 25,000 to 130,000), containing the carbamyl phosphate synthetase gene (AF154830.1); and

FIG. 10 is a Mondrian of BAC A049839.

Methods and Apparatus for Predicting, Confirming, Annotating, and Displaying Functional Regions From Genomic Sequence Data

FIG. 1 is a flow chart illustrating in broad outline a process for predicting functional regions from genomic sequence, confirming and characterizing the functional activity of such regions experimentally, and then associating and displaying the information so obtained in meaningful and useful relationship to the original sequence data.

The initial input into process 10 of the present invention is drawn from one or more databases 100 containing genomic sequence data. Because genomic sequence is usually obtained from subgenomic fragments, the sequence data typically will be stored in a series of records corresponding to these subgenomic sequenced fragments. Some fragments will have been catenated to form larger contiguous sequences ("contigs"); others will not. A finite percentage of sequence data in the database will typically be erroneous, consisting *inter alia* of vector sequence, sequence created from aberrant cloning events, sequence of artificial polylinkers, and sequence that was erroneously read.

Each sequence record in database 100 will minimally contain as annotation a unique sequence identifier (accession number), and will typically be annotated further to identify the date of accession, species of origin, and depositor. Because database 100 can contain nongenomic sequence, each sequence will typically be annotated further to permit query for genomic sequence. Chromosomal origin, optionally with map location, can also be present. Data can be, and over time increasingly will be, further annotated with additional information, in part through use of the present invention, as described below. Annotation can be present within the data records, in information external to database 100 and linked to the records thereto, or through a combination of the two.

Databases useful as genomic sequence database 100 in the present invention include GenBank, and particularly include several divisions thereof, including the htgs(draft), NT (nucleotide, command line), and NR (nonredundant) divisions. GenBank is produced by the National Institutes of Health and is maintained by the National Center for Biotechnology Information (NCBI). Databases of genomic sequence from species other than human, such as mouse, rat, Arabidopsis, *C. elegans*, *C.*

brigsii, *Drosophila*, zebra fish, and other higher eukaryotic organisms will also prove useful as genomic sequence database 100.

Genomic sequence obtained by query of genomic sequence database 100 is then input into one or more processes 200 for identification of regions therein that are predicted to have a biological function as specified by the user. Such functions include, but are not limited to, encoding protein, regulating transcription, regulating message transport after transcription into mRNA, regulating message splicing after transcription into mRNA, of regulating message degradation after transcription into mRNA, and the like. Other functions include directing somatic recombination events, contributing to chromosomal stability or movement, contributing to allelic exclusion or X chromosome inactivation, and the like.

The particular genomic sequence to be input into process 200 will depend upon the function for which relevant sequence is to be identified as well as upon the approach chosen for such identification. Process step 200 can be iterated to identify different functions within a given genomic region. In such case, the input often will be different for the several iterations.

Sequences predicted to have the requisite function by process 200 are then input into process 300, where a subset of the input sequences suitable for experimental confirmation is identified. Experimental confirmation can involve physical and/or bioinformatic assay. Where the subsequent experimental assay is bioinformatic, rather than physical, there are fewer constraints on the sequences that can be tested, and in this latter case therefore process 300 can output the entirety of the input sequence.

The subset of sequences output from process 300 is then used in process 400 for experimental verification and characterization of the function predicted in process 200, which experimental verification can, and often will, include both physical and bioinformatic assay.

Process 500 annotates the sequence data with the functional information obtained in the physical and/or bioinformatic assays of process 400. Such annotation can be done using any technique that usefully relates the functional information to the sequence, as, for example, by incorporating the functional data into the sequence data record itself, by linking records in a hierarchical or relational database, by linking to external databases, by a combination thereof, or by other means well known within the database arts. The data can even be submitted for incorporation into databases maintained by others, such as GenBank, which is maintained by NCBI.

As further noted in FIG. 1, additional annotation can be input into process 500 from external sources 600.

The annotated data is then displayed in process 800, either before, concomitantly with, or after optional storage 700 on nontransient media, such as magnetic disk, optical disc, magnetooptical disk, flash memory, or the like.

FIG. 1 shows that the experimental data output from process 400 can be used in each preceding step of process 10: e.g., facilitating identification of functional sequences in process 200, facilitating identification of an experimentally suitable subset thereof in process 300, and facilitating creation of physical and/or informational substrates for, and performance of subsequent assay, of functional sequences in process 400.

Information from each step can be passed directly to the succeeding process, or stored in permanent or interim form prior to passage to the succeeding process. Often, data will be stored after each, or at least a plurality, of such process steps. Any or all process steps can be automated.

FIG. 2 further elaborates the prediction of functional sequence within genomic sequence according to process 200.

Genomic sequence database 100 is first queried 20 for genomic sequence.

The sequence required to be returned by query 20 will depend, in the first instance, upon the function to be identified.

For example, genomic sequences that function to encode protein can be identified *inter alia* using gene prediction approaches, comparative sequence analysis approaches, or combinations of the two. In gene prediction analysis, sequence from one genome is input into process 200 where at least one, preferably a plurality, of algorithmic methods are applied to identify putative coding regions. In comparative sequence analysis, by contrast, corresponding, *e.g.*, syntenic, sequence from a plurality of sources, typically a plurality of species, is input into process 200, where at least one, possibly a plurality, of algorithmic methods are applied to compare the sequences and identify regions of least variability.

The exact content of query 20 will also depend upon the database queried. For example, if the database contains both genomic and nongenomic sequence, perhaps derived from multiple species, and the function to be determined is protein coding regions in human genomic sequence, the query will accordingly require that the sequence returned be genomic and derived from humans.

Query 20 can also incorporate criteria that compel return of sequence that meets operative requirements of the subsequent analytical method. Alternatively, or in addition, such operative criteria can be enforced in subsequent preprocess step 24.

For example, if the function sought to be identified is protein coding, query 20 can incorporate criteria that return from genomic sequence database 100 only those sequences present within contigs sufficiently long as to have obviated substantial fragmentation of any given exon among a plurality of separate sequence fragments.

Such criteria can, for example, consist of a required minimal individual genomic sequence fragment length, such as 10 kb, more typically 20 kb, 30 kb, 40kb, and preferably 50 kb or more, as well as an optional further or alternative requirement that sequence from any given clone, such as a bacterial artificial chromosome ("BAC"), be presented in no more than a finite maximal number of fragments, such as no more than 20 separate pieces, more typically no more than 15 fragments, even more typically no more than about 10 - 12 fragments.

Results using the present invention have shown that genomic sequence from bacterial artificial chromosomes (BACs) is sufficient for gene prediction analysis according to the present invention if the sequence is at least 50 kb in length, and if additionally the sequence from any given BAC is presented in fewer than 15, and preferably fewer than 10, fragments. Accordingly, query 20 can incorporate a requirement that data accessioned from BAC sequencing be in fewer than 15, preferably fewer than 10, fragments.

An additional criterion that can be incorporated into the query can be the date, or range of dates, of sequence accession. Although the process has been described above as if genomic sequence database 100 were static, it is of course understood that the genomic sequence databases need not be static, and indeed are typically updated on a frequent, even hourly, basis. Thus, as further described in Examples 1 and 2, *infra*, it is possible to query the database for newly added sequence, either newly added after an absolute date, or newly added relative to a prior analysis performed using the methods and apparatus of the present invention. In this way, the process herein described can incorporate a dynamic, temporal component.

One utility of such temporal limitation is to identify, from newly accessioned genomic sequence, the presence of novel genes, particularly those not previously identified by EST sequencing (or other sequencing efforts that are similarly based upon gene expression). As further described in Example 1, such an approach has shown that newly accessioned human genomic sequence, when analyzed for sequences that function to encode protein, readily identifies genes that are novel over those in existing EST and other expression databases. This makes the methods of the present invention extremely powerful gene discovery tools. And as would be appreciated, such gene discovery can be performed using genomic sequence from species other than human.

If query 20 incorporates multiple criteria, such as above-described, the multiple criteria can be performed as a series of separate queries or as a single query, depending in part upon the query language, the complexity of the query, and other considerations well known in the database arts.

If query 20 returns no genomic sequence meeting the query criteria, the negative result can be reported by process 22, and process 200 (and indeed, entire process 10) ended 23, as shown. Alternatively, or in addition to report and termination of the initial inquiry, a new query 20 can be generated that takes into account the initial negative result.

When query 20 returns sequence meeting the query criteria, the returned sequence is then passed to optional preprocessing 24, suitable and specific for the desired analytical approach and the particular analytical methods thereof to be used in process 25.

Preprocessing 24 can include processes suitable for many approaches and methods thereof, as well as processes specifically suited for the intended subsequent analysis.

Preprocessing 24 suitable for most approaches and methods will include elimination of sequence irrelevant to, or that would interfere with, the subsequent analysis. Such sequence includes repetitive sequence, such as Alu repeats and LINE elements, vector sequence, artificial sequence, such as artificial polylinkers, and the like. Such removal can readily be performed by identification and subsequent masking of the undesired sequence.

Identification can be effected by comparing the genomic sequence returned by query 20 with public or private databases containing known repetitive sequence, vector sequence, artificial sequence, and other artifactual sequence. Such comparison can readily be done using programs well known in the art, such as CROSS_MATCH, or by proprietary sequence comparison programs the engineering of which is well within the skill in the art.

Alternatively, or in addition, undesirable, including artifactual, sequence can be identified algorithmically without comparison to external databases and thereafter removed. For example, synthetic polylinker sequence can be identified by an algorithm that identifies a significantly higher than average density of known restriction sites. As another example, vector sequence can be identified by algorithms that identify nucleotide or codon usage at variance with that of the bulk of the genomic sequence.

Once identified, undesired sequence can be removed. Removal can usefully be done by masking the undesired sequence as, for example, by converting the specific nucleotide references to one that is unrecognized by the subsequent bioinformatic algorithms, such as "X". Alternatively, but at present less preferred, the undesired sequence can be excised from the returned genomic sequence, leaving gaps.

Preprocessing 24 can further include selection from among duplicative sequences of that one sequence of highest quality. Higher quality can be measured as a lower percentage of, fewest number of, or least densely clustered occurrence of ambiguous nucleotides, defined as those nucleotides that are identified in the genomic sequence using symbols indicating ambiguity. Higher quality can also or alternatively be valued by presence in the longest contig.

Preprocessing 24 can, and often will, also include formatting of the data as specifically appropriate for passage to the analytical algorithms of process 25. Such formatting can and typically will include, *inter alia*, addition of a unique sequence identifier, either derived from the original accession number in genomic sequence database 100, or newly applied, and can further include additional annotation. Formatting can include conversion from one to another sequence listing standard, such as conversion to or from FASTA or the like, depending upon the input expected by the subsequent process.

Preprocessing, which can be optional depending upon the function desired to be identified and the informational requirements of the methods for effecting such identification, is followed by sequence processing 25, where sequences with the desired function are identified within the genomic sequence.

As mentioned above, such functions can include, but are not limited to, encoding protein, regulating transcription, regulating message transport after transcription into mRNA, regulating message splicing after transcription, of regulating message degradation, and the like. Other functions include directing somatic recombination events, contributing to chromosomal stability or movement, contributing to allelic exclusion or X chromosome inactivation, or the like.

The methods of the present invention are particularly useful for gene discovery, that is, for identifying, from genomic sequence, regions that function to encode genes, and in a particularly useful embodiment, for identifying regions that function to encode genes not hitherto identified by expression-based or directed cloning and sequencing. In conjunction with verification using the novel single exon microarrays of the present invention, as further described below, the methods herein described become powerful gene discovery tools.

Accordingly, in a preferred embodiment of the present invention, process 25 is used to identify putative coding regions. Two preferred approaches in process 25 for identifying sequence that encodes putative genes are gene prediction and comparative sequence analysis.

Gene prediction can be performed using any of a number of algorithmic methods, embodied in one or more software programs, that identify open reading frames (ORFs) using a variety of heuristics, such as GRAIL, DICTION, and GENEFINDER. Comparative sequence analysis similarly can be performed using any of a variety of known programs that identify regions with lower sequence variability.

As further described in Example 1, below, gene finding software programs yield a range of results. For the newly accessioned human genomic sequence input in Example 1, for example, GRAIL identified the greatest percentage of genomic sequence as putative coding region, 2% of the data analyzed; GENEFINDER was second, calling 1%; and DICTION yielded the least putative coding region, with 0.8% of genomic sequence called as coding region.

Increased reliability can be obtained when consensus is required among several such methods. Although discussed herein particularly with respect to exon calling, consensus among methods will in general increase reliability of predicting other functions as well.

Thus, as indicated by query 26, sequence processing 25, optionally with preprocessing 24, can be repeated with a different method, with consensus among such iterations determined and reported in process 27.

Process 27 compares the several outputs for a given input genomic sequence and identifies consensus among the separately reported results. The consensus itself, as well as the sequence meeting that consensus, is then stored in process 29a, displayed in process 29b, and/or output to process 300 for subsequent identification of a subset thereof suitable for assay.

Multiple levels of consensus can be calculated and reported by process 27. For example, as further described in Example 1, *infra*, process 27 can report consensus as between all specific pairs of methods of gene prediction, as consensus among any one or more of the pairs of methods of gene prediction, or as among all of the gene prediction algorithms used. Thus, in Example 1, process 27 reported that GRAIL and GENEFINDER programs agreed on 0.7% of genomic sequence, that GRAIL and DICTION agreed on 0.5% of genomic sequence, and that the three programs together agreed on 0.25% of the data analyzed. Put another way, 0.25% of the genomic sequence was identified by all three of the programs as containing putative coding region.

Furthermore, consensus can be required among different approaches to identifying a chosen function.

For example, if the function desired to be identified is coding of protein sequence, and a first used approach to exon calling is gene prediction, the process can be repeated on the same input sequence, or subset thereof, with another approach, such as comparative sequence analysis. In such a case, where comparative sequence analysis follows gene prediction, the comparison can be performed not only on genomic nucleic acid sequence, but additionally or alternatively can be performed on the predicted amino acid sequence translated from the ORFs prior identified by the gene prediction approach.

Although shown as an iterative process, the multiple analyses required to achieve consensus can be done in series, in parallel, or some combination thereof.

Predicted functional sequence, optionally representing a consensus among a plurality of methods and approaches for determination thereof, is passed to process 300 for identification of a subset thereof for functional assay.

In the preferred embodiment of the methods of the present invention, wherein the function sought to be identified is protein coding, process 300 is used to identify a subset thereof suitable for experimental verification by physical and/or bioinformatic approaches.

For example, putative ORFs identified in process 200 can be classified, or binned, bioinformatically into putative genes. This binning can be based *inter alia* upon consideration of the average number of exons/gene in the species chosen for analysis, upon density of exons that have been called on the genomic sequence, and other empirical rules. Thereafter, one or more among the gene-specific ORFs can be chosen for subsequent use in gene expression assay.

Where such subsequent gene expression assay uses amplified nucleic acid, considerations such as desired amplicon length, primer synthesis requirements, putative exon length, sequence GC content, existence of possible secondary structure, and the like can be used to identify and select those ORFs that appear most likely successfully to amplify. Where subsequent gene expression assay relies upon nucleic acid hybridization, whether or not using amplified product, further considerations involving hybridization stringency can be applied to identify that subset of sequences that will most readily permit sequence-specific discrimination at a chosen hybridization and wash stringency. One particular such consideration is avoidance of putative exons that span repetitive sequence; such sequence can hybridize spuriously to nonspecific message, reducing specific signal in the hybridization.

For bioinformatic assay, there are fewer constraints on the sequences that can be tested experimentally, and in this latter case therefore process 300 can output the entirety of the input sequence.

The subset of sequences identified by process 300 as suitable for use in assay is then used in process 400 to create the physical and/or informational substrate for experimental verification of the predictions made in process 200, and thereafter to assay those substrates.

As mentioned, the methods of the present invention are particularly useful for identifying potential coding regions within genomic sequence. In a preferred embodiment of process 400, therefore, the expression of the sequences predicted to encode protein is verified. The combination of the predictive and experimental methods provides a powerful gene discovery engine.

Thus, in another aspect, the present invention provides methods and apparatus for verifying the expression of putative genes identified within genomic sequence. In particular, the invention provides a novel method of verifying gene expression in which expression of predicted ORFs is measured and confirmed using a novel type of nucleic acid microarray, the genome-derived single exon nucleic acid microarrays of the present invention.

Putative ORFs as predicted by a consensus of gene calling, particularly gene prediction, algorithms in process 200, and as further identified as suitable by process 300, are amplified from genomic DNA using the polymerase chain reaction (PCR). Although PCR is conveniently used, other amplification approaches can also be used.

Amplification schemes can be designed to capture the entirety of each predicted ORF in an amplicon with minimal additional (that is, intronic or intergenic) sequence. Because ORFs predicted from human genomic sequence using the methods of the present invention differ in length, such an approach results in amplicons of varying length.

However, most predicted ORFs are shorter than 500 bp in length, and although amplicons of at least about 100 or 200 base pairs can be immobilized as probes on nucleic acid microarrays, early experimental results using the methods of the present invention have suggested that longer amplicons, at least about 400 or 500 base pairs, are more effective. Furthermore, certain advantages derive from application to the microarray of amplicons of defined size.

Therefore, amplification schemes can alternatively, and preferably, be designed to amplify regions of defined size, preferably at least about 300, 400 or 500 bp, centered about each predicted ORF. Such an approach results in a population of amplicons of limited size diversity, but that typically contain intronic and/or intergenic nucleic acid in addition to putative ORF.

Conversely, somewhat fewer than 10% of ORFs predicted from human genomic sequence according to the methods of the present invention exceed 500 bp in length. Portions of such extended ORFs, preferably at least about 300,400 or 500 bp in length, can be amplified. However, it has been discovered that the percentage success at amplifying pieces of such ORFs is low, and that such putative exons are more effectively amplified when larger fragments, at least about 1000 or 1500 bp, and even as large as 2000 bp are amplified.

The putative ORFs selected in process 300 are thus input into one or more primer design programs, such as PRIMER3 (available online for use at http://www-genome.wi.mit.edu/cgi-bin/primer/), with a goal of amplifying at least about 500 base pairs of genomic sequence centered within or about ORFs predicted to be no more than about 500 bp, or at least about 1000 - 1500 bp of genomic sequence for ORFs predicted to exceed 500 bp in length, and the primers synthesized by standard techniques. Primers with the requisite sequences can be purchased commercially or synthesized by standard techniques.

Conveniently, a first predetermined sequence can be added commonly to the ORF-specific 5' primer and a second, typically different, predetermined sequence commonly added to each 3' ORF-unique primer. This serves to immortalize the amplicon, that is, serves to permit further amplification of any amplicon using a single set of primers complementary respectively to the common 5' and common 3' sequence elements. The presence of these "universal" priming sequences further facilitates later sequence verification, providing a sequence common to all amplicons at which to prime sequencing reactions. The common 5' and 3' sequences further serve to add a cloning site should any of the ORFs warrant further study.

Such predetermined sequence is usefully at least about 10, 12 or 15 nt in length, and usually does not exceed about 25 nt in length. The "universal" priming sequences used in the examples presented *infra* were each 16 nt long.

The genomic DNA to be used as substrate for amplification will come from the eukaryotic species from which the genomic sequence data had originally been obtained, or a closely related species, and can conveniently be prepared by well known techniques from somatic or germline tissue or cultured cells of the organism. See, *e.g.*, Short Protocols in Molecular Biology : A Compendium of Methods from Current Protocols in Molecular Biology, Ausubel et al. (eds.), 4th edition (April 1999), John Wiley & Sons (ISBN: 047132938X) and Maniatis *et al.*, Molecular Cloning : A Laboratory Manual, 2nd edition (December 1989), Cold Spring Harbor Laboratory Press (ISBN: 0879693096). Many such prepared genomic DNAs are available commercially, with the human genomic DNAs additionally having certification of donor informed consent.

Although the intronic and intergenic material flanking putative coding regions in the amplicons could potentially interfere with hybridizations during microarray experiments, we have found, surprisingly, that differential expression ratios are not significantly affected. Rather, the predominant effect of exon size is to alter the absolute signal intensity, rather than its ratio. Equally surprising, the art had suggested that single exon probes would not provide sufficient signal intensity for high stringency hybridization analyses; we find that such probes not only provide adequate signal, but have substantial advantages, as herein described.

After partial purification, as by size exclusion spin column, with or without confirmation as to amplicon quality as by gel electrophoresis, each amplicon (single exon probe) is disposed in an array upon a support substrate.

Methods for creating microarrays by deposition and fixation of nucleic acids onto support substrates are well known in the art (Reviewed by Schena et al., see above).

Typically, the support substrate will be glass, although other materials, such as amorphous or crystalline silicon or plastics. Such plastics include polymethylacrylic, polyethylene, polypropylene, polyacrylate, polymethylmethacrylate, polyvinylchloride, polytetrafluoroethylene, polystyrene, polycarbonate, polyacetal, polysulfone, celluloseacetate, cellulosenitrate, nitrocellulose, or mixtures thereof, can also be used. Typically, the support will be rectangular, although other shapes, particularly circular disks and even spheres, present certain advantages. Particularly advantageous alternatives to glass slides as support substrates for array of nucleic acids are optical discs, as described in WO 98/12559.

The amplified nucleic acids can be attached covalently to a surface of the support substrate or, more typically, applied to a derivatized surface in a chaotropic agent that facilitates denaturation and adherence by presumed noncovalent interactions, or some combination thereof.

Robotic spotting devices useful for arraying nucleic acids on support substrates can be constructed using public domain specifications (The MGuide, version 2.0, http://cmgm.stanford.edu/pbrown/mguide/index.html), or can conveniently be purchased from commercial sources (MicroArray GenII Spotter and MicroArray GenIII Spotter, Molecular Dynamics, Inc., Sunnyvale, CA). Spotting can also be effected by printing methods, including those using ink jet technology.

As is well known in the art, microarrays typically also contain immobilized control nucleic acids. For controls useful in providing measurements of background signal for the genome-derived single exon microarrays of the present invention, a plurality of *E. coli* genes can readily be used. As further described in Example 1, 16 or 32 *E. coli* genes suffice to provide a robust measure of background noise in such microarrays.

As is well known in the art, the amplified product disposed in arrays on a support substrate to create a nucleic acid microarray can consist entirely of natural nucleotides linked by phosphodiester bonds, or alternatively can include either nonnative nucleotides, alternative internucleotide linkages, or both, so long as complementary binding can be obtained in the hybridization. If enzymatic amplification is used to produce the immobilized probes, the amplifying enzyme will impose certain further constraints upon the types of nucleic acid analogs that can be generated.

Although particularly described herein as using high density microarrays constructed on planar substrates, the methods of the present invention for confirming the expression of ORFs predicted from genomic sequence can use any of the known types of microarrays, as herein defined, including lower density planar arrays, and microarrays on nonplanar, nonunitary, distributed substrates.

For example, gene expression can be confirmed using hybridization to lower density arrays, such as those constructed on membranes, such as nitrocellulose, nylon, and positively-charged derivatized nylon membranes. Further, gene expression can also be confirmed using nonplanar, bead-based microarrays such as are described in Brenner *et al.*, *Proc. Natl. Acad. Sci. USA* 97(4):166501670 (2000); U.S. Patent No. 6,057,107; and U.S. Patent No. 5,736,330. In theory, a packed collection of such beads provides in aggregate a higher density of nucleic acid probe than can be achieved with spotting or lithography techniques on a single planar substrate.

Planar microarrays on solid substrates, however, provide certain useful advantages, including high throughput and compatibility with existing readers. For example, each standard microscope slide can include at least 1000, typically at least 2000, preferably 5000 and upto 10,000 - 50,000 or more nucleic acid probes of discrete sequence. The number of sequences deposited will depend on their required application.

Each putative gene can be represented in the array by a single predicted ORF. Alternatively, genes can be represented by more than one predicted ORF. For purposes of measuring differential splicing, more than one predicted ORF will be provided for a putative gene. And as is well known in the art, each probe of defined sequence, representing a single predicted ORF, can be deposited in a plurality of locations on a single microarray to provide redundancy of signal.

The genome-derived single exon microarrays described above differ in several fundamental and advantageous ways from microarrays presently used in the gene expression art, including (1) those created by deposition of mRNA-derived nucleic acids, (2) those created by *in situ* synthesis of oligonucleotide probes, and (3) those constructed from yeast genomic DNA.

Most nucleic acid microarrays that are in use for study of eukaryotic gene expression have as immobilized probes nucleic acids that are derived — either directly or indirectly — from expressed message. As discussed above, it is common, for example, for such microarrays to be derived from cDNA/EST libraries, either from those previously described in the literature, see Lennon *et al.*, or from the *de novo* construction of "problem specific" libraries targeted at a particular biological question, R.S. Thomas *et al.*, *Cancer Res.* (in press). Such microarrays are herein collectively denominated "EST microarrays".

Such EST microarrays by definition can measure expression only of those genes found in EST libraries, shown herein to represent only a fraction of expressed genes. Furthermore, such libraries — and thus microarrays based thereupon – are biased by the tissue or cell type of message origin, by the expression levels of the respective genes within the tissues, and by the ability of the message successfully to have been reverse-transcribed and cloned.

Thus, as further discussed in Example 1, the methods of the present invention enable sequences that do not appear in EST or other expression databases to be determined - subsequently arrayed for expression measurements could not, therefore, have been represented as probes on an EST microarray. And as further demonstrated in the examples, *infra*, the remaining population of genes identified from genomic sequence by the methods of the present invention – that is, the one third of sequences that had previously been accessioned in EST or other expression databases – are biased toward genes with higher expression levels.

Representation of a message in an EST and/or cDNA library depends upon the successful reverse transcription, optionally but typically with subsequent successful cloning, of the message. This introduces substantial bias into the population of probes available for arraying in EST microarrays.

In contrast, neither reverse transcription nor cloning is required to produce the probes arrayed on the genome-derived single exon microarrays of the present invention. And although the ultimate deposition of a probe on the genome-derived single exon microarray of the present invention depends upon a successful amplification from genomic material, *a priori* knowledge of the sequence of the desired amplicon affords greater opportunity to recover any given probe sequence recalcitrant to amplification than is afforded by the requirement for successful reverse transcription and cloning of unknown message in EST approaches.

Thus, the genome-derived single exon microarrays of the present invention present a far greater diversity of probes for measuring gene expression, with far less bias, than do EST microarrays presently used in the art.

As a further consequence of their ultimate origin from expressed message, the probes in EST microarrays often contain poly-A (or complementary poly-T) stretches derived from the poly-A tail of mature mRNA. These homopolymeric stretches contribute to cross-hybridization, that is, to a spurious signal occasioned by hybridization to the homopolymeric tail of a labeled cDNA that lacks sequence homology to the gene-specific portion of the probe.

In contrast, the probes arrayed in the genome-derived single exon microarrays of the present invention lack homopolymeric stretches derived from message polyadenylation, and thus can provide more specific signal. Typically, at least about 50, 60 or 75% of the probes on the genome-derived single exon microarrays of the present invention lack homopolymeric regions consisting of A or T, where a homopolymeric region is defined for purposes herein as stretches of 25 or more, typically 30 or more, identical nucleotides.

A further distinction, which also affects the specificity of hybridization, is occasioned by the typical derivation of EST microarray probes from cloned material. Because much of the probe material disposed as probes on EST microarrays is excised or amplified from plasmid, phage, or phagemid vectors, EST microarrays typically include a fair amount of vector sequence, more so when the probes are amplified, rather than excised, from the vector.

In contrast, the vast majority of probes in the genome-derived single exon microarrays of the present invention contain no prokaryotic or bacteriophage vector sequence, having been amplified directly or indirectly from genomic DNA. Typically, therefore, at least about 50, 60, 70 or 80% or more of individual exon-including probes disposed on a genome-derived single exon microarray of the present invention lack vector sequence, and particularly lack sequences drawn from plasmids and bacteriophage. Preferably, at least about 85, 90 or more than 90% of exon-including probes in the genome-derived single exon microarray of the present invention lack vector sequence. With attention to removal of vector sequences through preprocessing 24, percentages of vector-free exon-including probes can be as high as 95 - 99%. The substantial absence of vector sequence from the genome-derived single exon microarrays of the present invention results in greater specificity during hybridization, since spurious cross-hybridization to a probe vector sequence is reduced.

As a further consequence of excision or amplification of probes from vectors in construction of EST microarrays, the probes arrayed thereon often contain artificial sequence, derived from vector polylinker multiple cloning sites, at both 5' and 3' ends. The probes disposed upon the genome-derived single exon microarrays need have no such artificial sequence appended thereto.

As mentioned above, however, the ORF-specific primers used to amplify putative ORFs can include artificial sequences, typically 5' to the ORF-specific primer sequence, useful for "universal" (that is, independent of ORF sequence) priming of subsequent amplification or sequencing reactions. When such "universal" 5' and/or 3' priming sequences are appended to the amplification primers, the probes disposed upon the genome-derived single exon microarray will include artificial sequence similar to that found in EST microarrays. However, the genome-derived single exon microarray of the present invention can be made without such sequences, and if so constructed, presents an even smaller amount of nonspecific sequence that would contribute to nonspecific hybridization.

Yet another consequence of typical use of cloned material as probes in EST microarrays is that such microarrays contain probes that result from cloning artifacts, such as chimeric molecules containing coding region of two separate genes. Derived from genomic material, typically not thereafter cloned, the probes of the genome-derived single exon microarrays of the present invention lack such cloning artifacts, and thus provide greater specificity of signal in gene expression measurements.

A further consequence of the cloned origin of probes on many EST microarrays is that the individual probes often have disparate sizes, which can cause the optimal hybridization stringency to vary among probes on a single microarray. In contrast, as discussed above, the probes arrayed on the genome-derived single exon microarrays of the present invention can readily be designed to have a narrow distribution in sizes, with the range of probe sizes no greater than about 10% of the average size, typically no greater than about 5% of the average probe size.

Because of their origin from fully- or partially-spliced message, probes disposed upon EST arrays will often include multiple exons. The percentage of such exon-spanning probes in an EST microarray can be calculated, on average, based upon the predicted number of exons/gene for the given species and the average length of the immobilized probes. For human genes, the near-complete sequence of human chromosome 22, Dunham *et al.*, *Nature* 402(6761):489-95 (1999), predicts that human genes average 5.5 exons/gene. Even with probes of 200 - 500 bp, the vast majority of human EST microarray probes include more than one exon.

In contrast, by virtue of their origin from algorithmically identified ORFs in genomic sequence, the probes in the genome-derived single exon microarrays of the present invention can consist of individual exons. Thus, in contrast to EST microarrays, at least about 50, 60, 70, 75, 80, 85, 95 or 99% of probes deposited in the genome-derived microarray of the present invention consist of, or include, no more than one predicted ORF.

This provides the ability, not readily achieved using EST microarrays, to use the genome-derived single exon microarrays of the present invention to measure tissue-specific expression of individual exons, which in turn allows differential splicing events to be detected and characterized, and in particular, allows the correlation of differential splicing to tissue-specific expression patterns.

Furthermore, the exons that are represented in EST microarrays are often biased toward the 3' or 5' end of their respective genes, since sequencing strategies used for EST identification are so biased. In contrast, no such 3' or 5' bias necessarily inheres in the selection of exons for disposition on the genome-derived single exon microarrays of the present invention.

Conversely, the probes provided on the genome-derived single exon microarrays of the present invention typically, but need not necessarily, include intronic and/or intergenic sequence that is absent from EST microarrays, which are derived from mature mRNA. Typically, at least about 50, 60, 70, 80 or 90% of the exon-including probes on the genome-derived single exon microarrays of the present invention include sequence drawn from noncoding regions. As discussed above, the additional presence of noncoding region does not significantly interfere with measurement of gene expression, and provides the additional opportunity to assay prespliced RNA, and thus measure such phenomena such as nuclear export control.

The genome-derived single exon microarrays of the present invention are also quite different from *in situ* synthesis microarrays, where probe size is severely constrained by inadequacies in the photolithographic synthesis process.

Typically, probes arrayed on *in situ* synthesis microarrays are limited to a maximum of about 25 bp. As a well known consequence, hybridization to such chips must be performed at low stringency. In order, therefore, to achieve unambiguous sequence-specific hybridization results, the *in situ* synthesis microarray requires substantial redundancy, with concomitant programmed arraying for each probe of probe analogues with altered (*i.e.*, mismatched) sequence.

In contrast, the longer probe length of the genome-derived single exon microarrays of the present invention allows much higher stringency hybridization and wash. Typically, therefore, exon-including probes on the genome-derived single exon microarrays of the present invention average at least about 100, 200, 300, 400 or 500 bp in length. By obviating the need for substantial probe redundancy, this approach permits a higher density of probes for discrete exons or genes to be arrayed on the microarrays of the present invention than can be achieved for *in situ* synthesis microarrays.

A further distinction is that the probes in *in situ* synthesis microarrays typically are covalently linked to the substrate surface. In contrast, the probes disposed on the genome-derived microarray of the present invention typically are, but need not necessarily be, bound noncovalently to the substrate.

Furthermore, the short probe size on *in situ* microarrays causes large percentage differences in the melting temperature of probes hybridized to their complementary target sequence, and thus causes large percentage differences in the theoretically optimum stringency across the array as a whole.

In contrast, the larger probe size in the microarrays of the present invention create lower percentage differences in melting temperature across the range of arrayed probes.

A further significant advantage of the microarrays of the present invention over *in situ* synthesized arrays is that the quality of each individual probe can be confirmed before deposition. In contrast, the quality of probes cannot be assessed on a probe-by-probe basis for the *in situ* synthesized microarrays presently being used.

The genome-derived single exon microarrays of the present invention are also distinguished over, and present substantial benefits over, the genome-derived microarrays from lower eukaryotes such as yeast. Lashkari *et al.*, *Proc. Natl. Acad. Sci. USA* 94:13057-13062 (1997).

Only about 220 - 250 of the 6100 or so nuclear genes in *Saccharomyces cerevisiae* — that is, only about 4 - 5% — have standard, spliceosomal, introns, Lopez *et al.*, *Nucl. Acids Res.* 28:85-86 (2000); Spingola *et al.*, *RNA* 5(2):221-34 (1999). Furthermore, the entire yeast genome has already been sequenced. These two facts permit the ready amplification and disposition of single-ORF amplicons on such microarray without the requirement for antecedent use of gene prediction and/or comparative sequence analyses.

Thus, a significant aspect of the present invention is the ability to identify and to confirm expression of predicted coding regions in genomic sequence drawn from eukaryotic organisms that have a higher percentage of genes having introns than do yeast such as *Saccharomyces cerevisiae*, particularly in genomic sequence drawn from eukaryotes in which at least about 10, 20 or 50% of protein-encoding genes have introns. In preferred embodiments, the methods and apparatus of the present invention are used to identify and confirm expression of novel genes from genomic sequence of eukaryotes in which the average number of introns per gene is at least about one, two or three or more.

After the physical substrate is prepared, experimental verification of predicted function is performed.

In a preferred embodiment of the present invention, where the function sought to be identified in genomic sequence is protein coding, experimental verification is performed by measuring expression of the putative ORFs, typically through nucleic acid hybridization experiments, and in particularly preferred embodiments, through hybridization to genome-derived single exon microarrays prepared as above- described.

Expression is conveniently measured and expressed for each probe in the microarray as a ratio of the expression measured concurrently in a plurality of mRNA sources, according to techniques well known in the microarray art, *Reviewed in* Schena et al., and as further described in Example 2, below. The mRNA source for the reference against which specific expression is measured can be drawn from a homogeneous mRNA source, such as a single cultured cell-type, or alternatively can be heterogeneous, as from a pool of mRNA derived from multiple tissues and/or cell types, as further described in Example 2, *infra*.

mRNA can be prepared by standard techniques, see Ausubel et al. and Maniatis *et al.*, or purchased commercially. The mRNA is then typically reverse-transcribed in the presence of labeled nucleotides: the index source (that in which expression is desired to be measured) is reverse transcribed in the presence of nucleotides labeled with a first label, typically a fluorophore (fluorochrome; fluor; fluorescent dye); the reference source is reverse transcribed in the presence of a second label, typically a fluorophore, typically fluorometrically-distinguishable from the first label. As further described in Example 2, *infra*, Cy3 and Cy5 dyes prove particularly useful in these methods. After partial purification of the index and reference targets, hybridization to the probe array is conducted according to standard techniques, typically under a coverslip.

After wash, microarrays are conveniently scanned using a commercial microarray scanning device, such as a Gen3 Scanner (Molecular Dynamics, Sunnyvale, CA). Data on expression is then passed, with or without interim storage, to process 500, where the results for each probe are related to the original sequence.

Often, hybridization of target material to the genome-derived single exon microarray will identify certain of the probes thereon as of particular interest. Thus, it is often desirable that the user be able readily to obtain sufficient quantities of an individual probe, either for subsequent arrayed deposition upon an additional support substrate, often as part of a microarray having a plurality of probes so identified, or alternatively or additionally as a solitary solid-phase or solution-phase probe, for further use.

Thus, in another aspect, the present invention provides compositions and kits for the ready production of nucleic acids identical in sequence to, or substantially identical in sequence to, probes on the genome-derived single exon microarrays of the present invention.

In this aspect, a small quantity of each probe is disposed, typically without attachment to substrate, in a spatially-addressable ordered set, typically one per well of a microtiter dish. Although a 96 well microtiter plate can be used, greater efficiency is obtained using higher density arrays, such as are provided by microtiter plates having 384, 864, 1536, 3456, 6144, or 9600 wells, and although microtiter plates having physical depressions (wells) are conveniently used, any device that permits addressable withdrawal of reagent from fluidly-noncommunicating areas can be used.

In this aspect of the invention, therefore, a fluidly noncommunicating addressable ordered set of individual probes, corresponding to those on a genome-derived single exon microarray, is provided, with each probe in sufficient quantity to permit amplification, such as by PCR. As earlier mentioned, the ORF-specific 5' primers used for genomic amplification can have a first common sequence added thereto, and the ORF-specific 3' primers used for genomic amplification can have a second, different, common sequence added thereto, thus permitting, in this preferred embodiment, the use of a single set of 5' and 3' primers to amplify any one of the probes from the amplifiable ordered set.

Each discrete amplifiable probe can also be packaged with amplification primers, solutes, buffers, *etc.*, and can be provided in dry (*e.g.*, lyophilized) form or wet, in the latter case typically with addition of agents that retard evaporation.

In another aspect of the present invention, a genome-derived single-exon microarray is packaged together with such an ordered set of amplifiable probes corresponding to the probes, or one or more subsets of probes, thereon. In alternative embodiments, the ordered set of amplifiable probes is packaged separately from the genome-derived single exon microarray.

In some embodiments, the microarray and/or ordered probe set are further packaged with recordable media that provide probe identification and addressing information, and that can additionally contain annotation information, such as gene expression data. Such recordable media can be packaged with the microarray, with the ordered probe set, or with both.

If the microarray is constructed on a substrate that incorporates recordable media, such as is described in international patent application no. WO 98/12559, then separate packaging of the genome-derived single exon microarray and the bioinformatic information is not required.

The amount of amplifiable probe material should be sufficient to permit at least one amplification sufficient for subsequent hybridization assay.

Although the use of high density genome-derived microarrays on solid planar substrates is presently a preferred approach for the physical confirmation and characterization of the expression of sequences predicted to encode protein, other types of microarrays (as herein defined) can also be used.

Furthermore, as earlier mentioned, experimental verification of the function predicted from genomic sequence in process 200 can be bioinformatic, rather than, or additional to, physical verification.

For example, where the function desired to be identified is protein coding, the predicted ORFs can be compared bioinformatically to sequences known or suspected of being expressed.

Thus, the sequences output from process 300 (or process 200), can be used to query expression databases, such as EST databases, SNP ("single nucleotide polymorphism") databases, known cDNA and mRNA sequences, SAGE ("serial analysis of gene expression") databases, and more generalized sequence databases that allow query for expressed sequences. Such query can be done by any sequence query algorithm, such as BLAST ("basic local alignment search tool"). The results of such query — including information on identical sequences and information on nonidentical sequences that have diffuse or focal regions of sequence homology to the query sequence – can then be passed directly to process 500, or used to inform analyses subsequently undertaken in process 200, process 300, or process 400.

Experimental data, whether obtained by physical or bioinformatic assay in process 400, is passed to process 500 where it is usefully related to the sequence data itself, a process colloquially termed "annotation". Such annotation can be done using any technique that usefully relates the functional information to the sequence, as, for example, by incorporating the functional data into the record itself, by linking records in a hierarchical or relational database, by linking to external databases, or by a combination thereof. Such database techniques are well within the skill in the art.

The annotated sequence data can be stored locally, uploaded to genomic sequence database 100, and/or displayed 800.

The methods and apparatus of the present invention rapidly produce functional information from genomic sequence. Coupled with the escalating pace at which sequence now accumulates, the rapid pace of sequence annotation produces a need for methods of displaying the information in meaningful ways.

FIG. 3 shows visual display 80 presenting a single genomic sequence annotated according to the present invention. Because of its nominal resemblance to artistic works of Piet Mondrian, visual display 80 is alternatively described herein as a "Mondrian".

Each of the visual elements of display 80 is aligned with respect to the genomic sequence being annotated (hereinafter, the "annotated sequence"). Given the number of nucleotides typically represented in an annotated sequence, representation of individual nucleotides would rarely be readable in hard copy output of display 80. Typically, therefore, the annotated sequence is schematized as rectangle 89, extending from the left border of display 80 to its right border. By convention herein, the left border of rectangle 89 represents the first nucleotide of the sequence and the right border of rectangle 89 represents the last nucleotide of the sequence.

As further discussed below, however, the Mondrian visual display of annotated sequence can serve as a convenient graphical user interface for computerized representation, analysis, and query of information stored electronically. For such use, the individual nucleotides can conveniently be linked to the X axis coordinate of rectangle 89. This permits the annotated sequence at any point within rectangle 89 readily to be viewed, either automatically – for example, by time-delayed appearance of a small overlaid window upon movement of a cursor or other pointer over rectangle 89 – or through user intervention, as by clicking a mouse or other pointing device at a point in rectangle 89.

Visual display 80 is generated after user specification of the genomic sequence to be displayed. Such specification can consist of or include an accession number for a single clone (*e.g.*, a single BAC accessioned into GenBank), wherein the starting and stopping nucleotides are thus absolutely identified, or alternatively can consist of or include an anchor or fulcrum point about which a chosen range of sequence is anchored, thus providing relative endpoints for the sequence to be displayed. For example, the user can anchor such a range about a given chromosomal map location, gene name, or even a sequence returned by query for similarity or identity to an input query sequence. When visual display 80 is used as a graphical user interface to computerized data, additional control over the first and last displayed nucleotide will typically be dynamically selectable, as by use of standard zooming and/or selection tools.

Field 81 of visual display 80 is used to present the output from process 200, that is, to present the bioinformatic prediction of those sequences having the desired function within the genomic sequence. Functional sequences are typically indicated by at least one rectangle 83 (83a, 83b, 83c), the left and right borders of which respectively indicate, by their X-axis coordinates, the starting and ending nucleotides of the region predicted to have function.

Where a single bioinformatic method or approach identifies a plurality of regions having the desired function, a plurality of rectangles 83 is disposed horizontally in field 81. Where multiple methods and/or approaches are used to identify function, each such method and/or approach can be represented by its own series of horizontally disposed rectangles 83, each such horizontally disposed series of rectangles offset vertically from those representing the results of the other methods and approaches.

Thus, rectangles 83a in FIG. 3 represent the functional predictions of a first method of a first approach for predicting function, rectangles 83b represent the functional predictions of a second method and/or second approach for predicting that function, and rectangles 83c represent the predictions of a third method and/or approach.

Where the function desired to be identified is protein coding, field 81 is used to present the bioinformatic prediction of sequences encoding protein. For example, rectangles 83a can represent the results from GRAIL or GRAIL II, rectangles 83b can represent the results from GENEFINDER, and rectangles 83c can represent the